REMARKS/ARGUMENTS

In response to the Office Action of September 11, 2006, Applicants request re-examination and reconsideration of this application for patent pursuant to 35 U.S.C. 132.

Claim Status/Support for Amendments

Claims 1-18 and 22-49 have been cancelled. New claims 50-57 have been added. Claims 19-21 are withdrawn from consideration. It is understood that claims 19-21, drawn to the non-elected invention of Group III, will remain pending, albeit withdrawn from consideration on the merits at this time. Applicants retain the right to present the non-elected claims 19-21 in a divisional application. Claims 19-21 and 50-57 remain pending in the instant application.

No new matter has been added with the addition of new claims 50-57.

New claims 50-57 incorporate the subject of originally filed claims. Claim 50 incorporates the subject matter of original claims 6 and 16. Claim 51 incorporates the subject matter of original claims 26 and 31. Claim 52 incorporates the subject matter of original claims 6, 9, 10 and 16. Claim 53 incorporates the subject matter of original claims 26, 29 and 31. Claim 54 incorporates the subject matter of original claims 13. Claim 55 incorporates the

subject matter of original claims 22 and 30. Claim 56 incorporates the subject matter of original claims 13-15. Claim 57 incorporates the subject matter of original claims 22, 25 and 30.

* Please note that the Examiner's comments from the Office Action reiterated herein are single spaced to clearly delineate the Examiner's comments from Applicants' comments.

Request for Rejoining of Claims

Considering that claims 54-57 are carried out using the pharmaceutical composition of the elected Group II invention (claims 50-53), a search of these claims would encompass this specific pharmaceutical composition. Applicants respectfully request that the Examiner consider rejoining claims 54-57 in the instant application, which are currently drawn to non-elected methods (Group I), with claims of the elected Group II (claims 50-53) under the decision in *In re Ochiai* (MPEP 2116.01), upon the Examiner's determination that claims of the elected invention (claims 50-53) are allowable and in light of the overlapping search. If the pharmaceutical composition of the elected Group II invention is found to be novel, methods (claims 54-57) limited to its use should also be found novel.

Information Disclosure Statement

The Examiner notes that Ingman et al. (Page 2, of the IDS filed 9/05) has been considered. However, in the absence of an alignment with a sequence of the instant application, the Examiner cannot assess the relevance of the reference.

The Ingman reference is a GeneBank entry (Accession Number AAK17620) disclosing the amino acid sequence of human NADH dehydrogenase subunit 2 (ND2). ND2 is present in the post-synaptic density (PSD)and acts as an adaptor protein anchoring Src to the NMDAR complex. See the instant specification as originally filed at page 9, lines 2-23; page 18, lines 7-11; Example 1 and SEQ ID NO:9.

Objections to the Claims

Claims 9-10, 17-18, 29, and 33-35, as presented on June 30, 2006, stand objected to under 37 CFR 1.75 c, as allegedly being of improper dependent form for failing to further limit the subject matter of a previous claim. Applicant is required to cancel the claim(s), or amend the claim(s) to place the claim(s) in proper dependent form, or rewrite the claim(s) in independent form. In the above claims, Applicant recites an intended use (i.e. for cells of a CNS) which does not further structurally limit the composition claimed.

Claims 9-10, 17-18, 29, and 33-35 have been cancelled. The subject matter of original claims 9, 10 and 29 has been rewritten in independent form as new claims 52, 53, 56 and 57.

Thus, Applicants have addressed the Examiner's objection and respectfully request that this objection to the claims now be withdrawn.

Claim 6, as presented on June 30, 2006, stands objected to because of the following alleged informality: It contains a reference to "SUDAPI", without first disclosing the meaning of the acronym. In order to make the description of the invention more clear, the first claim that mentions these acronyms should fully express the phrase, and be followed by parentheses, which identify the acronym to be used in the following claim(s). Amendment of claim 6 to include "SUDAPI" in parentheses right after the term "Src unique domain anchoring protein inhibitor" would overcome the objection. Appropriate correction is required.

Applicants note that claim 1, as originally presented, recites "SUDAPI" in parentheses right after the term "Src unique domain anchoring protein inhibitor".

Claim 6 has been cancelled. New claims 50-57 do not recite the term "SUDAPI".

Thus, Applicants have addressed the Examiner's objection and respectfully request that this objection now be withdrawn.

Rejections under 35 USC 112, first paragraph

Claims 6-10, 12, 16-18, 26-29, 31-35, 40-43, and 47-49, as presented on June 30, 2006, stand rejected under 35 USC 112, first paragraph, because the specification, while being enabling for a pharmaceutical composition of polypeptide SEQ ID NO:2, allegedly does not reasonably provide enablement for **"modifying"** NMDA receptor interaction with Src comprising at least one **"SUDAPI"** or for a pharmaceutical composition of SEQ ID NO:1.

The Examiner asserts that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention commensurate in scope with these claims.

The factors considered when determining if the disclosure satisfies the enablement requirement and whether any necessary experimentation is "undue" include, but are not limited to: 1) nature of the invention, 2) state of the prior art, 3) relative skill of those in the art, 4) level of predictability in the art,

5) existence of working examples, 6) breadth of the claims, 7) amount of direction or guidance by the inventor, and 8) quantity of experimentation needed to make or use the invention. In re Wands, 859 F.2d 731, 737, 8USPQ2d 1400, 1402 (Federal Circuit 1988).

The nature of the invention is a generic "SUDAPI" inhibitor that works by *modifying* an *intracellular* interaction between Src and NMDA in neurons of the brain or peripheral nervous system. The generic inhibitor must be capable of transport across the blood-brain barrier and further transport across the plasma membrane of the target neuron. The invention is complex because the specification provides *one* sequence coupled to HIV-Tat that *inhibits* Src-NMDA interaction, but the invention claimed is to *any* (*structurally undefined*) compound that *modifies* Src-NMDA interaction, and is capable of delivery into the intracellular environment of neurons in the brain and peripheral nervous system (PNS).

The prior art does not recognize a "SUDAPI", and does not recognize compounds that enhance Src-NMDA interaction (which is reasonably encompassed by "modifying"). The prior art recognizes coupling of HIV-Tat to polypeptides (but not generic SUDAPI compounds) for brain/neuron delivery (see Schwarze et al 1999). However, the prior art does not recognize that polypeptides such as SEQ ID NO:1 can be delivered effectively to the brain and intracellular environment of a neuron without HIV-Tat or another known protein transport domain. Therefore, a "pharmaceutical composition" comprising SEQ ID NO:1 is not supported.

The working examples show that the composition comprising SEQ ID NO:2 is useful for *inhibiting* interaction between Src and NMDA, and for treating pain in animal model of pain. The working examples do not support any other molecule, or generic "SUDAPI" for modifying or inhibiting the interaction between Src and NMDA.

The breadth of the claims is such that they include unknown and undisclosed "SUDAPIs" that "modify" Src-NMDA receptor interaction. Further, there is no guidance as to how to make a SUDAPI, let alone make a SUDAPI that "modifies" Src-NMDA receptor interaction.

Therefore, the Examiner concludes that given the complex nature of the invention, the lack of support in the prior art, and the lack of examples or guidance, one skilled in the art would not be able to make or use the invention as currently claimed.

Applicants respectfully disagree with the Examiner's determination.

Claims 6-10, 12, 16-18, 26-29, 31-35, 40-43, and 47-49 have been cancelled. New claims 50-53 have been added which encompass the subject matter of these claims as originally filed.

Although Applicants believe that the instant specification fully enables compositions that modify interaction between Src and the NMDAR complex, in the interest of compact and efficient prosecution, the claims have been amended to recite a pharmaceutical composition for inhibiting NMDAR complex interaction with non-receptor tyrosine kinase Src and/or inhibiting non-receptor tyrosine kinase Src in cells consisting of SEQ ID NO:2 combined with a pharmaceutically acceptable solution.

Thus, Applicants respectfully request that this rejection under 35 USC 112, first paragraph (scope of enablement) now be withdrawn.

Claims 6-10, 26-29, and 32-35, as presented on June 30, 2006, stand rejected under 35 USC 112, first paragraph, as allegedly failing to comply with the written description requirement. The claim(s) contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The claims are drawn to unknown and undisclosed molecules called "SUDAPIs". The claims do not require that the compounds possess any particular biological activity except modifying the interaction between Src and NMDA, nor do generic "SUDAPIs" have any particular conserved structure, or other disclosed distinguishing feature. Therefore, there are no clear structural limitations on the complex of molecules claimed. Thus, the claims are drawn to a broad functionally-defined genus that lacks any structural

definition.

To provide evidence of possession of a claimed genus, the specification must provide sufficient distinguishing identifying characteristics of the genus. In the instant application, no such distinctions have been made. One polypeptide has been disclosed, SEQ ID NO:2, which is not sufficient to characterize the broad class of compounds claimed. The factors to be considered include disclosure of complete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, methods of making the claimed product, or any combination thereof. Applicant has described one polypeptide.

Accordingly, in the absence of sufficient recitation of distinguishing identifying characteristics, the specification does not provide adequate written description of the claimed genus.

Vas-Cath Inc. v. Mahurkar, 19USPQ2d 1111, clearly states that "applicants must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention*. The invention is, for the purposes of the "written description" inquiry, *whatever is now claimed.*" (See page 1117). The specification does not "clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed." (See *Vas-Cath* at page 1116). As discussed above, the skilled artisan cannot envision the detailed chemical structure of the encompassed genus of compounds and therefore conception is not achieved until reduction to practice has occurred. Adequate written description requires more than a mere statement that it is part of the invention. The compound itself is required. See *Fiers v. Revel*, 25 USPQ2d 1601 at 1601 (CAFC 1993) and *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ2d 1016.

One cannot describe what one has not conceived. See *Fiddes v. Baird*, 30 USPQ2d 1481 at 1483. In *Fiddes*, claims directed to mammalian FGF's were found to be unpatentable due to a lack of written description for that broad class. The specification provided only the bovine sequence.

Therefore, the Examiner concludes that only polypeptides described in the specification have sufficient written description, and not generic "SUDAPIs". Applicant is reminded that *Vas-Cath* makes clear that the written description provision of 35 USC 112 is severable from its enablement provision (see page 1115).

Applicants respectfully disagree with the Examiner's determination.

Claims 6-10, 12, 16-18, 26-29, 31-35, 40-43, and 47-49 have

been cancelled. New claims 50-53 have been added which encompass the subject matter of these claims as originally filed.

Although Applicants believe that the instant specification fully describes "SUDAPIs", in the interest of compact and efficient prosecution, the claims have been amended to recite a pharmaceutical composition consisting of SEQ ID NO:2 combined with a pharmaceutically acceptable solution.

Thus, Applicants respectfully request that this rejection under 35 USC 112, first paragraph (written description) now be withdrawn.

Rejection under 35 USC 112, second paragraph

Claims 6-10, 12, 16-18, 33-35, and 40-43, as presented on June 30, 2006, stand rejected under 35 USC 112, second paragraph, as being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 6 and 16 describe a pharmaceutical composition for "modifying" Src-NMDA receptor interaction comprising a "SUDAPI". "Modifying" reasonably includes inhibiting and activating or facilitating. Thus the claims encompass any manner of altering said interaction. However, Applicant defines "SUDAPI" as "Src unique domain anchoring protein inhibitor" which reasonably does not activate or facilitate the interaction it simultaneously inhibits. Claims 7-10, 17-18, 33-35, and 40-43 are rejected because they depend from the indefinite claims 6 and 16.

Applicants respectfully disagree with the Examiner's determination.

Claims 6-10, 12, 16-18, 33-35 and 40-43 have been cancelled.

New claims 50-53 have been added which encompass the subject matter of these claims as originally filed.

Although Applicants believe that the described composition is distinctly claimed as "modifying" an interaction between the NMDAR and non-receptor tyrosine kinase Src, in the interest of compact and efficient prosecution, the claims have been amended to recite a composition for inhibiting an interaction between the NMDAR and non-receptor tyrosine kinase Src.

Accordingly, Applicants have now clarified the metes and bounds of the claims and respectfully request that the above-discussed rejection under 35 USC 112, second paragraph be withdrawn.

Rejection under 35 USC 102/103

Claims 6, 9-10, 26 and 29, as presented on June 30, 2006, stand rejected under 35 USC 102(b) as allegedly being anticipated by or, in the alternative, under 35 USC 103(a) as obvious over Gingrich et al. Society for Neuroscience Abstract, entitled "ND2, A mitochondrially-encoded protein, interacts with Src Kinase at the NMDA receptor complex" 2001.

The Examiner asserts that Gingrich et al teach Src peptide 40-58 corresponding to the instant peptide of SEQ ID NO:1, which is Src peptide 39-47. Gingrich et al report that Src40-58 disrupts Src tyrosine kinase modulation of NMDA receptor activity and indicate that it does by inhibiting Src-NMDA interaction (see abstract). Therefore, the Examiner concludes that Gingrich is a SUDAPI. The Src peptide is disclosed as used in an epitope-tagged binding assay. This assay (and others) was undoubtedly performed in an aqueous solution, which meets the limitation of "pharmaceutically acceptable solution" in the rejection under 102(b). Alternatively, it would be obvious to solvate the peptide in an aqueous solution

because that is how nearly all peptides are stored in solution.

Applicants respectfully disagree with the Examiner's assertions.

Original claims 6, 9-10, 26 and 29 have been cancelled. New claims 50-53 incorporate the subject matter of these claims.

It has been established that in order for a claim to be anticipated each and every element as set forth in the claim must be found, either expressly or inherently described in a single prior art reference (see MPEP 2131).

Applicants respectfully assert that the peptide of the claimed pharmaceutical composition is distinct from the peptide disclosed by Gingrich et al.

New claims 50-53 are drawn to a pharmaceutical composition consisting of SEQ ID NO:2. SEQ ID NO:2 is a peptide having amino acid residues corresponding approximately to amino acid positions 40-49 of the Src unique domain combined with the carrier peptide, HIV-TAT (see the instant specification at page 18, line 15 to page 19, line 18). Amino acid residues 40-49 of the Src unique domain were identified as the specific binding region by examining the interaction of different subpeptides, which were derived from amino acid residues 40-58 of the Src unique domain, with ND2 (see the instant specification as originally filed at page 47, Example 6 and Figure 6).

Furthermore, Applicants note that the phrase "consisting of" is closed language and excludes any element, step or ingredient not specified in the claims (see MPEP 2111.03). Thus, the scope of the claims is limited to a peptide having the amino acid residues of SEQ ID NO:2.

Accordingly, the peptide of the pharmaceutical composition, as currently recited in the claims, has a sequence corresponding to amino acid residues 40-49 of the Src unique domain combined with or fused to the HIV TAT transduction domain. This peptide (SEQ ID NO:2) specifically binds ND2 to inhibit the interaction of Src with the NMDAR.

The abstract of Gingrich et al. discloses a peptide, corresponding to amino acid residues 40-58 of the unique domain of Src, that binds the ND2 protein and inhibits the interaction of Src with the NMDAR to downregulate Src-mediated NMDAR function. However, Gingrich does not disclose the specific binding region at amino acid residues 40-49 because this binding region was identified by data gathered from experimentation performed after the presentation of the cited poster/abstract (see Declaration of Dr. Ken Pelkey attached hereto). Furthermore, Gingrich et al. do not teach any peptide fusions, including fusions with the HIV transduction domain (TAT). SEQ ID NO:2 has elements that are not found in the sequence of Gingrich et al., i.e. HIV TAT transuction

domain. The peptide of Gingrich et al. has elements that are not found in SEQ ID NO:2, i.e. amino acid residues 50-58 corresponding the unique domain of Src. Thus, the peptide of the claimed pharmaceutical composition (SEQ ID NO:2) does not have a structure identical to that of the peptide of Gingrich et al.

Accordingly, since the claimed pharmaceutical composition is distinct from the peptide disclosed by Gingrich et al., the reference can not be said to describe each and every element in claims 50-53.

A disclosure of a genus in the prior art is not necessarily a disclosure of every species (*In re Baird* 29 USPQ2d 1550). When prior art teaches a range overlapping or touching, the claimed range anticipates only if the prior art range discloses the claimed range with sufficient specificity (MPEP 2131.03).

The range of amino acid residues of the Src unique domain covered by the prior art (abstract of Gingrich et al.) overlaps that of the described peptide (SEQ ID NO:2). The cited prior art discloses a peptide corresponding to amino acid residues 40-58 of the unique domain of the Src protein. The claimed invention discloses a peptide corresponding to amino acid residues 40-49 of the unique domain of the Src protein. However, the instant Applicants show that subpeptides, derived from the 40-58 amino acid sequence, do not exhibit equivalent binding properties. For

example, the subpeptide having amino acid residues 49-58 does not bind ND2 (see Example 6 and Figure 6). Thus, one of skill in the art would not be able to discern which portion of Gingrich's 40-58 amino acid sequence was the specific binding portion without further experimentation.

Thus, Applicants respectfully submit that the cited abstract does not anticipate the claimed invention, as the range of amino acid residues covered is not disclosed with sufficient specificity.

Accordingly, Applicants respectfully submit that they have now pointed out how the pharmaceutical composition as instantly claimed distinguishes over the peptide taught by Gingrich et al. and request that this rejection under 35 USC 102/103 now be withdrawn.

Rejection under 35 USC 103(a)

Claims 6-10, 26-29 and 32-35, as presented on June 30, 2006, stand rejected under 35 USC 103(a) as allegedly being unpatentable over Gingrich et al as applied to claims above, and further in view of Schwarze et al (1999).

Gingrich discloses a "SUDAPI" polypeptides that blocks the interaction between Src and NMDAR as described above in under 35 USC 102(b).

The Examiner asserts that Gingrich does not disclose coupling the peptide to HIV-TAT protein as that the polypeptide can be carried across the blood brain barrier or into the intracellular space of neurons.

Schwarze et al. teach delivery of a protein, β-galactosidase, into brain cells of a mouse by conjugation with the HIV-TAT protein (-YGRKKKRRQRRR-' see page 1572, bibliography, #7). See Figure 4, wherein Schwarze et al show that TAT collects in the cell bodies of CA-3 pyramidal neurons (page 1572).

The Examiner asserts that one of ordinary skill in the art

would be motivated to combine the method of Gingrich et al with the HIV TAT sequence of Schwarze et al because it would allow delivery of the competitive peptide Sec unique domain binding sequence (SEQ ID NO:1) to neurons in the brain and CNS to block the interaction between Src and NMDAR.

The motivational statement is further given by Schwarze stating "These results open new possibilities for direct delivery of proteins into patients in the context of protein therapy...[abstract]".

One of ordinary skill would have a reasonable expectation of success in delivery of the peptide to the intracellular environment of CNS/PNS neurons because Schwarze et al suggest that HIV TAT would be useful for treatment of *any* therapeutic protein, including those that must be targeted to the brain (abstract).

Applicants respectfully disagree with the Examiner's assertions.

Both the teachings of the instant invention and of Gingrich et al. are applied as above in the section covering the rejection under 35 USC 102/103.

Schwarze et al. teach *in vivo* protein transduction using the TAT transduction domain (see abstract). For example, Schwarze et al. show delivery of TAT-β-Gal into the brain cells of a mouse (page 1571, center column and Figure 4). Schwarze et al. do not teach transduction of peptides corresponding to the unique domain of the Src protein, including SEQ ID NO:2.

The Examiner asserts that one of ordinary skill in the art would be motivated to use the peptide disclosed by Gingrich et al. (amino acid residues 40-58 of the unique domain of Src) in the TAT delivery method of Schwarze et al. because it would allow delivery of the peptide into neurons in the brain. The Examiner concludes that one of ordinary skill in the art would have a reasonable expectation of success in delivery of the peptide because Schwarze et al. suggest HIV TAT would be useful for treatment for *any*

therapeutic protein, including those that must be targeted to the brain.

Applicants respectfully submit that the Examiner's conclusion appears to be based on the premise that protein transduction using HIV-TAT is always successful for transporting any peptide into any cell without a loss of biological activity/function. However, one of skill in the art recognizes that this is not the reality. For example, Schwarze et al. disclose less than ideal results by mentioning that a previous attempt to transduce β-Gal chemically cross-linked to the TAT PTD into mice resulted in sporadic and weak β-Gal activity in a limited number of tissues, with no activity detected in the kidney or brain (page 1571, right column).

Additionally, the TAT peptide is known to transduce virtually all types of cells and thus, has limited specificity. Many potential protein therapies, such as those for cancer, require targeted delivery to specific cells. Thus, in contrast to the Examiner's conclusion, HIV TAT may not be useful for any therapeutic protein.

Fusion with TAT may get a peptide into a cell, but the peptide may not function as predicted once inside the cell.

For example, a limitation of transduction (using TAT peptides) in terms of delivery of biologically active proteins is the requirement for the partial or even complete unfolding (denaturing)

of the protein that occurs during the transduction process (see page 4296, center column and Table 1 of Stephens et al. PNAS 98(8):4295-4296 2001; attached hereto and labeled reference #1). Thus, in many instances, a transduced protein must re-fold intracellularly to re-gain its biological activity.

Additionally, the physical fusion of a peptide to TAT may deactivate the peptide's biological function; i.e. TAT may mask a binding site. For example, in the instant case, it is possible that fusion of the 40-58 peptide with TAT may inhibit binding of ND2 and thus limit the peptide's ability to modify NMDAR activation. Thus, even if one of ordinary skill in the art were to use the peptide disclosed by Gingrich et al. with the TAT transduction process of Schwarze et al., one would not be able to discern if the peptide would be transduced and biologically functional. One would have to try each of numerous transductions until one arrived at a successful result.

Accordingly, Applicants respectfully submit that the Examiner has applied an improper "obvious to try" rationale in support of this obviousness rejection (see MPEP 2145 X B).

In light of all of the above remarks, Applicants respectfully submit that the Examiner has failed to establish a *prima facie* case of obviousness and further contend that a biologist of ordinary skill in the art, having the cited references (Gingrich and

Schwarze) in front of him/her would not have the information and motivation necessary to arrive at Applicants' invention.

Thus, it is respectfully submitted that the combination of the peptide of Gingrich et al. with the process of Schwarze fails to reasonably teach or suggest to one of ordinary skill in biology/medicine the elements of Applicants' pharmaceutical composition (peptide consisting of SEQ ID NO:2 combined with the HIV TAT transduction domain) as specifically set forth in the claims presented herein.

Accordingly, Applicants respectfully submit that the claimed pharmaceutical composition distinguishes over the prior art and respectfully request that this rejection under 35 USC 103(a) now be withdrawn.

CONCLUSION

In light of the foregoing remarks and amendments to the claims, it is respectfully submitted that the Examiner will now find the claims of the application allowable. Favorable reconsideration of this application is courteously requested.

Respectfully submitted,

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The many ways to cross the plasma membrane

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The transfer into living cells of macromolecules, which monitor or modify molecule-specific intracellular processes, provides an efficient way to study the temporal and spatial regulation of protein systems that underlie basic cellular functions. Several methods have been developed for this purpose. Each of them has its characteristic advantages and disadvantages with respect to cell viability, transfer efficiency, general applicability, and technical requirements. We discuss current methodologies for the introduction of macromolecules, notably proteins, into cells in light of a new paper by Walev *et al.* (1) in a recent issue of PNAS in which a new protocol is provided for the reversible permeabilization of cells by using streptolysin-O. The function and interaction of many more novel proteins identified by the genome projects worldwide will have to be analyzed in the future. Therefore, transfer techniques that are easy to use, inexpensive, and suitable for automation such as the one described by Walev *et al.* (1) will become of superior importance.

Worldwide genomic and cDNA sequencing projects are now identifying new molecules every day. The genomic sequences of the most widely studied species, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, have recently been completed. The full genomic sequence of *Homo sapiens* will become available soon. An enormous task, and a great challenge, will now be to relate these sequences to functional data that may subsequently open new avenues of research toward the cure of diseases. Together with the methodological progress made in the past two decades in expressing and manipulating cloned genes in the test tube, these sequence data provide us with the necessary molecular tools to understand the connectivity and spatial organization of protein systems in their native environment, the living cell.

Common strategies in modern cell and molecular biology, which aim toward the identification of the role of an unknown protein in the context of the intact cell, involve the introduction into living cells of molecules that allow one to monitor the localization or biochemical state of the protein (e.g., by fluorescence microscopy-based methods, ref. 2) or that interfere with its function (e.g., dominant negative mutants). Several such molecule transfer methods have been developed for this purpose. Direct transfer methods introduce the molecule of interest precisely into the cell. In carrier-mediated transfer methods the molecule of interest is loaded into or coupled to a general carrier that can cross the plasma membrane itself and thereby helps the passenger to enter the cell. A third class of transfer methods uses chemicals, bacterial toxins, or electrical pulses to transiently permeabilize the plasma membrane. The molecule transfer occurs then via diffusion through the pores formed.

Each of the methods has its characteristic advantages and disadvantages with respect to cell viability, transfer efficiency, general applicability, and technical requirements (see Table 1).

Direct Transfer Methods

The principal and only widely used direct and most efficient of all transfer methods is glass capillary microinjection, which was first reported about 30 years ago (3, 4). Transfer efficiencies and survival rates of up to 100% can be reached. Glass micropipettes with a fine tip of less than 0.5 μm are used to inject the sample of interest into the cell nucleus or cytoplasm of adherent cells. Microinjection is direct, and quantitative and conjection of several distinct molecules at fixed stoichiometric ratios is possible. A huge variety of molecules can be injected, and even injection of entire organelles has been reported (5). Furthermore, the molecules of interest can be injected at well-defined stages of the cell cycle, and cell culture conditions can be modified before, during, or after injection. Unfortunately microinjection is technically demanding. Although the specialized equipment needed, including prepulled micropipettes, is now commercially available (see for example ref. 6), a lengthy training period is required until reproducible results are obtained on a routine basis. A further drawback of classical microinjection methodologies is that only a few cells (of the order of 100–200) can be injected in one experiment, and thus analyses by biochemical methods or electron microscopy is difficult. There is also a limitation to the cell types that can be readily used for microinjection. Cultures that grow in suspension are obviously more difficult to use, as are those adherent cells that have only small volume nuclei or cytoplasm. However, computer-automated or semiautomated injection methods allow the injection of up to 1,500 cells per hour and advanced analysis of injected cells has become possible (6, 7). Future developments of such methods certainly will facilitate microinjection further, and it may become possible to even automate the systematic transfer of distinct molecules into a large number of cells at high throughput as would be necessary for large-scale genomic or proteomic projects.

Carrier-Mediated Transfer

In contrast to the direct transfer methods, a vast variety of protocols exist that use a cell-permeable molecule as a generic carrier or to which the molecules to be introduced into cells is coupled to (see Table 1). When the carrier enters the cell the molecule to be introduced enters the cell as well in a "piggyback" manner. Such an approach allows targeting of thousands or millions of cells at the same time, and thus allows, in contrast to transfer by microinjection, much more sophisticated subsequent analyses, such as immunoprecipitation and Western blot analysis, subcellular fractionation, or electron microscopy. Application of the "loaded" carriers to whole animals is possible and can thus be applied in gene therapy approaches. The protocols for carrier-mediated transfer are usually simple, and technically advanced equipment is not required. Several carriers have been developed commercially and are thus readily available. These techniques have, however, been optimized predominantly for transfer of DNA or RNA and are less characterized for their use in transfer of proteins or small cell regulatory molecules that usually cannot cross the plasma membrane.

An interesting naturally occurring carrier is the homeodomain of the *D. melanogaster* protein Antennapedia. This 60-aa peptide is able to enter live cells in an energy-independent process that is different from endocytosis and appears not to require cell surface receptors (8–10). A 16-aa fragment of this peptide (penetra-

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Table 1. Common transfer techniques: Advantages and disadvantages

Method	Ref(s).	Advantages	Disadvantages
Direct transfer			
Capillary microinjection	6	High efficiency (100%) and survival rate, quantitative, few restrictions on target cells and sample, compartment specific transfer	Limited number of cells can be injected, expensive and technically demanding
Carrier-mediated transfer			
Protein transduction	11	Receptor, energy and transport independent, simultaneous transfer to large number of cells, *in vivo* applications possible	Size restriction in molecules that can be transferred, transfer is accompanied by protein unfolding
Liposome-mediated transfer	18	High efficiency, little restrictions in sample size, variety of molecules can be transferred, *in vivo* applications possible	Interferes with lipid metabolism
Microprojectile bombardment	22, 38	Applicable to wide range of cells (including plant cells), surface receptor independent, excellent for *in vivo* gene therapy	Specialized equipment needed, works best for DNA transfection
Transfer by plasma membrane permeabilization			
Reconstituted systems	26, 28, 30	Exchange of whole cytosol possible, large number of target cells addressable, cheap and simple to use	Cells are semi-intact
SLO permeabilization	1	Works with variety of sample molecules, high efficiency, cells remain intact, large number of target cells addressable, cheap and simple to use, could be automated	Size restriction (100 kDa) of molecules that can be transferred
Electroporation	35, 36	Large number of target cells addressable, applicable to a wide range of cells (including plant cells), little sample restrictions, high efficiency	Requires specialized equipment, works best when cells are in suspension

tin-1), which is short enough to be easily synthetically synthesized, has been shown to retain the properties necessary for penetration. Unfortunately, attempts to routinely introduce large proteins or DNA molecules (>100 residues) have been difficult so far (11, 12). The penetratin system is also ineffective for the uptake of double-stranded DNA (8).

A further commercially available method is based on the VP22 protein of herpes simplex virus 1. The VP22 protein has a unique ability to translocate between mammalian cells. Expression of VP22 fusions in cells facilitates expression of a fusion protein that is exported from the transfected cells and is then translocated (transduced) into the nontransfected cells where it localizes to the nucleus (e.g., see ref. 13); however, the originally transfected cells show a principle localization of the fusion proteins to the cytoplasm. One must consider the localization of the proteins used in such experiments in relation to biological activity. It is possible to provide a homogenous population of cells by preparing cell lysates from the originally transfected cells and incubating this with a separate population. Of particular note are a number of recent publications concerning the transfer of VP22-GFP (green fluorescent protein) fusion proteins between cells (13–15). These studies consistently report difficulties in the detection of GFP fluorescence in cells to which the VP22-GFP fusion has been transferred (as opposed to the original, transfected cells); GFP fluorescence was not detectable in living cells and only weakly so when fixed (13–15). Although this is possibly due to the folding status of GFP, pH-dependent quenching, or some other form of quenching, it seems most likely that transfer of the protein was simply below the level of detection in these experiments (13).

A similar technology to VP22 is the use of the TAT protein from HIV-1. The TAT protein transduction domain is typically fused to the protein of interest and generated as a recombinant protein in bacteria (see ref. 11). After purification, the fusion proteins are used to transduce cells with high efficiency. The key limitation of this approach in terms of biological delivery of active proteins is the requirement for the partial or even complete unfolding of the protein that occurs during the transduction process (11). Despite this, many active enzymes have been successfully delivered as TAT fusions (see ref. 11). This approach, like that using VP22, has the benefit of being able to deliver large protein molecules of over 100 kDa. Indeed, 40-nm superparamagnetic iron particles were taken up efficiently when coupled to multiple copies of the TAT peptide sequence (16). The technique is also applicable to every eukaryotic cell line tested thus far with the exception of yeast. Even *Drosophila* has been shown to be susceptible by painting the eyes with a solution containing the fusion protein (11). The key to this technology would appear to be the refolding of the TAT fusion proteins within the cells.

Other more universal approaches of carrier-mediated transfer, which also allow transfer of larger molecules, use cationic lipids for introduction of DNA, RNA, or proteins into cells (17, 18). Most protocols use commercially available monocationic lipids such as DOTAP (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate), DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride), or DOSPA (2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate). In particular, success has been achieved with some of the newer formulations of these lipids (17). Unilamellar or multilamellar liposomes with a diameter between 100 and 400 nm are generated. The positive charges of the liposomes allow them to interact spontaneously with the negatively charged backbones of polynucleotides to form complexes. These can then interact with the negatively charged cell membrane, resulting in the delivery of the polynucleotides into the target cells. This approach eliminates the need for the generation of fusion proteins or chemical peptide protein coupling but is likely limited to acidic proteins that complex readily with the cationic lipids routinely used. Because the ability of these liposomes to incorporate water- or lipid-soluble molecules, liposomes also have

been used as a means to deliver pharmacologically active molecules to specific sites in the body (17, 19, 20). Liposome-mediated delivery needs to be interpreted with caution because significant changes in cellular metabolism can be induced, particularly with regard to lipid metabolism (21). A new generation of liposome-like transfection reagents has started appearing on the market recently. The Chariot reagent from Active Motif (Carlsbad, CA) is apparently specifically designed for protein transfection and boasts efficiencies of 60–85%. Such technologies are clearly a welcome development but await wider usage and independent testing before a thorough evaluation of their potential.

Introduction of probes into cells or tissues by microprojectile bombardment uses subcellular-sized particles, which are coated with DNA and accelerated at high velocities toward target cells (22, 38). Thousands of cells can be penetrated and transformed with one bombardment. The physical nature of the process makes it applicable to a wide range of cell and tissue types and a diversity of organisms. It has thus become one of the most widely used methods for plant transformation for which many other transfer methods fail because of the rigid nature of the cell wall. More recently, this method has been applied to deliver DNA to whole tissue *in vivo* (23) and thus represents a true alternative to viral vectors in gene therapy (24).

A major uncertainty in most carrier-mediated transfer methods is that efficiency varies with cell type (18) and usually is far below 100%. This poses problems in the interpretation of those experiments where the molecule introduced has inhibitory function. With a typical transfer efficiency of for example 30%, the maximum inhibition for the entire cell population analyzed will approach 30%, and thus the significance of results is unclear unless the transfer efficiency can be precisely determinced independently. Enrichment of successfully targeted cells, by, for example, flow cytometry, may help to overcome such problems.

Transfer by Transient Permeabilization of the Plasma Membrane

A third class of transfer methods uses detergents, pore-forming toxins, UV laser illumination, or short electrical pulses to permeabilize the plasma membrane. Transfer then occurs via passive diffusion through the pores. A critical parameter in these approaches is the survival rate of the cells. Experimental conditions, which generate sufficiently large pores for sufficient molecule transfer, coincide with increased cell death in a fraction of the cells. On the other hand, in the same experiment some cells are often not sufficiently permeabilized and thus the number of successfully permeabilized and vital cells is around 50% of the entire target cell population. Similar to the carrier-mediated transfer techniques, this poses a problem when inhibitory molecules are to be introduced.

Although it has been difficult to overcome these limitations, cell permeabilization by pore-forming toxins or detergents has been extensively used in the past in so-called "reconstituted systems" using "semi-intact" cells. In these methods cells are irreversibly permeabilized, which even allows exchange of the entire cytosol of the cells (25–30). This has clear advantages because the exogenously added cytosol can be immuno-depleted of interesting factors and the molecules to be introduced (e.g., mutants of the molecule of interest) are thus not in competition with the endogenous pool of the molecules of interest. Addition of exogenous components to assays have been extremely useful for studying many systems, for example, the recruitment of protein complexes to membranes. The ability to detect only the exogenously added, purified adaptor complexes against a background of endogenous protein greatly facilitated analysis of adaptor recruitment to membranes (31). Species-specific antibodies against bovine adaptors enabled specific detection in recruitment assays using rat cell lines (31). The major concern in using these methods remains that it has always been difficult to assess to which extent the semi-intact cells are a valid model for the intact cell. Clearly these approaches are an excellent step toward dissecting molecular processes; however, there is always a concern that these detergents also affect important cellular structures, such as the cytoskeleton, or membrane organelles and therefore semi-intact cell systems are by no means perfect models for the living cells.

Considerable progress has now been made to overcome these limitations by recent work using the pore-forming toxin streptolysin-O (SL-O) (1, 32–34). Under certain conditions cells can repair SL-O lesions (32, 33) and fluorescent proteins could be demonstrated to be taken up by cells treated with low doses of SL-O. Walev *et al.* (1) have succeeded now to provide very good evidence that proteins of up to 100 kDa can be taken up by SL-O-permeabilized cells without any loss of their function. The protocols developed allow about 50% of the treated cells to take up the molecule of interest and to survive for days without any obvious sign of lethality. Cell sorting may indeed be a suitable step to enrich for those cells that have taken up the molecules used. A key point to note from these experiments, as Walev *et al.* (1) note themselves, is the purity of the SL-O used for the studies. Furthermore, accurate estimation of cell permeabilization and recovery is essential to accurate interpretation of results. As with many of the carrier-mediated protocols described above, transient SL-O permeabilization was found to have an effective size limit in terms of efficient uptake. Although small proteins such as antibody Fab fragments and low molecular weight dextrans were efficiently taken up, Walev *et al.* found that molecules above ≈100 kDa were excluded from the permeabilized cells (whole molecular antibodies and high M_r dextrans). This finding is in a similar range to the majority of experiments using carrier-mediated methods.

Similar features to those obtained with these new developments also can be achieved by electroporation, which uses microsecond, high-voltage pulses to transiently permeabilize the plasma membrane (35, 36). Similar to the SL-O method about 50% of the treated cells survive and take up the molecule of interest. Unfortunately, in most applications cells need to be in solution for electroporation, which can be achieved by treatment with trypsin or chelators of metal ions in the case of adherent tissue culture cells. This, however, disrupts a number of important cellular relationships as cell cycle progression, cell adhcrence, and signal transduction, which are therefore difficult to he studied with electroporation.

As with all of the methods described here there are experiments that clearly benefit from one or another of these approaches in particular. For example, a quantitative study of delivery of antisense oligonucleotides to chronie myeloid leukaemia cells showed SL-O permeabilization or electroporation to be superior to liposome-mediated delivery (37). Indeed electroporation was found to give more reproducieble results than SL-O treatment, suggesting great variability in the efficacy of SL-O within individual experiments.

It is clear that further developments of this approach will be necessary in the future, but the method as it stands so far appears to combine the advantages of the semi-intact strategics as simplicity, possibility to exchange cytosolic factors with a high dcgree of surviving cells. This new protocol now allows exchange of cytosolic factors on a large scale by simple treatment of cells under conditions that keep cells intact.

Conclusions

None of the transfer techniques described above are of sufficient flexibility to cover all possible applications and demands of molecule transfer into living cells or tissues. Future developments should include, however, possibilities for automation of the methods to reach the throughput required for systematic large-scale genome projects. In this respect the method described by Walev *et al.* (1) may have great potential as it requires easily automated handling steps, only pipetting and washing of reagents and cells, and

does not require specialized equipment. In addition, it offers acceptable transfer efficiencies and most importantly permeabilized cells appear to remain intact. Increasing the throughput in capillary microinjection, which is so far unmatched in its transfer efficiency and flexibility in the kind of molecules that can be transferred, by further automization of the method may be another route to take.

As important as the improvements in terms of throughput will be to improve the possibilities to deliver genes, small molecules, drugs, or even proteins into tissues of whole bodies, because transfer of molecules *in vivo* will be of critical importance to translate the forthcoming wealth of information on individual genes by genome projects into animal models and therapies.

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The many ways to cross the plasma membrane

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